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JAACT SPECIAL ISSUE

Cephalic hedgehog expression is regulated directly by Sox17 in endoderm development of *Xenopus laevis*

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Abstract In early development of animals, hedgehog (Hh) genes function as morphogen in the axis determination and the organ formation. In *Xenopus*, three hedgehog genes, sonic (shh), banded (bhh), and cephalic (chh), were identified and might organize various tissues and organs in embryogenesis. Here, we report the spatial and temporal regulation of Xchh which is expressed in endoderm cells differentiating to digestive organs. Xchh expression in endoderm was inhibited by ectopic expression of the dominant-negative activin receptor, tAR. Moreover, a maternally inherited transcription factor VegT and its downstream regulators activated Xchh expression. These indicates that Xchh is regulated by the factor involved in the cascade originated from VegT via activin/nodal signals. Using the Sox17α-VP16-GR construct, we showed that Xchh expression might be induced directly by transcription factor Sox17.

Keywords Endoderm development · Hedgehog · Nodal · VegT · *Xenopus* · Xsox17

Abbreviations

bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
TGFβ	transforming growth factor β

Introduction

In embryogenesis, the pluripotent embryonic cells differentiate to various types of cells and become to show specific function in organ and in the whole body. In the process of cell differentiation, various factors secreted from the neighboring cells act as differentiation factors. In addition, to organize the differentiated cells into the functional tissue and organs, morphogens are necessary. Morphogens are defined as molecules produced in organizing center of tissue and possess capability to determine tissue and organ polarity in dose dependent manner. As one of the morphogens, hedgehog was identified firstly in fruit fly (Nüsslein-Volhard et al. 1980). Subsequently, in higher organisms, three or four hedgehog gene homologes were found and play important roles to form the proper tissues and organs. (reviewed by Hammerschmidt et al. 1997)

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In *Xenopus*, there are three hedgehog genes, sonic (Xshh), banded (Xbhh), and cephalic (Xchh) (Ekker et al. 1995), which are expressed at the confined regions and developmental stages during early embryogenesis. In early neurula embryo, Xshh are expressed in notochord cell and affects the adjacent neural tube cell to differentiate to floor plate cell (Echelard et al. 1993; Krauss et al. 1993; Roelink et al. 1994; Fan et al. 1994). Consequently, Xshh determines the direction of neural tissue along dorsal-ventral axis and the various neural cells, such as motorneuron, intermediate-neuron and sensory-neuron are originated in accordance with this direction. This indicates that the precise expression of morphogen at the proper region and period in embryo is a key to organize the animal body coordinately.

Molecular mechanism of Xshh expression in embryo has been studied. Xshh is induced by the cooperative effects of Xnr1 and Noggin (Ito et al. 2001), which are produced from the vegetal cells and Spemann organizer cells in embryo, respectively, through the intronic enhancer elements in its genome (unpublished data). Moreover, It is suggested that the transcription factor belonging to the forkhead family is induced by Xnr1 and operates to the intronic enhancer elements (unpublished data).

Another *Xenopus* hedgehog gene, Xchh, is expressed at pan-endoderm region in blastula embryo and later, its expression localizes at anterior edge of endoderm, suggesting that Xchh plays an important role in endoderm development and can relate to organize the endoderm tissue. Similar to shh, the localized expression of Xchh in embryo might lead to the proper formation of endodermal organs. The molecular mechanism of Xchh expression has not been solved yet.

In *Xenopus*, the differentiation of endoderm is thought to begin by the action of maternally inherited transcription factor VegT (Clements et al. 1999; Xanthos et al. 2001; Clements et al. 2003) which is synthesized during oogenesis and stored at presumptive endoderm region (vegetal region) of egg. After fertilization, VegT induces expression of zygotic transcription factor Sox7 directly (Zhang et al. 2003, 2005a), followed by induction of Xnr5/6 (Takahashi et al. 2000), which are early-activated *Xenopus* nodals (Zhang et al. 2003). Then, Xnr5/6 activates Xnr1/2/4 expressions (Joseph and Melton 1997) and finally, Xnr1/2/4 activate endoderm-specific transcription factors, Bix (Tada et al. 1998; Ecochard et al. 1998; Casey et al. 1999), Mix (Rosa 1989; Vize 1996; Lemaire et al. 1998), Mixer (Henry et al. 1998), GATA4/5/6 (Kelley et al. 1993; Jiang et al. 1996; Weber et al. 2000, Afouda et al. 2005) and Xsox17 (Hudson et al. 1997). Nodals are members of TGFβ superfamily and are capable to induce the differentiation of endoderm and mesoderm cells in dose dependent manner. Hence, the gradient of Nodal proteins could define the boundary between endoderm and mesoderm and also the positions of Nieuwkoop center and Spemann organizer.

In this report, we focused on the elucidation of the regulation mechanism of Xchh expression and examined which signal cascade could activate the expression of Xchh in endoderm.

Materials and methods

Embryo culture and manipulation

Xenopus laevis eggs were obtained from hormone (Gonadtropin 3000; Teikoku Zoki Co.)–stimulated females, fertilized, and cultured (Sive et al. 2000). Embryos were staged according to Nieuwkoop and Faber (1967). Animal cap and vegetal pole explants were isolated at the mid-blastula stage (stage 8.5) and cultured until desired stages in 1% MBS containing 1 mg/ml bovine serum albumin.

Capped mRNAs were synthesized in vitro from linearized plasmid templates encoding dominant-negative FGF receptor (XFD) (Amaya et al. 1991), dominant-negative activin receptor (tAR) (Hemmati-Brivanlou et al. 1992), dominant-negative BMP receptor (tBR) (Suzuki et al. 1994), BVg1 (Thomsen et al. 1993), and Xsox17α-en/VP16-GR (Hudson et al. 1997; Sinner et al. 2004) using mMessage mMachine kits (Ambion, Inc.).

Extraction of RNA and RT-PCR analysis

Total RNA was extracted from embryos and explants using phenol methods with minor modifications; after the phenol extraction step, the aqueous phase was added to an equal volume of 5 M ammonium acetate and then stored at 0°C to eliminate genomic DNA. For semi-quantitative RT-PCR, cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen).

Oligo-dT primed reverse-transcription was performed using 1 μg total RNA as a template. Each cDNA was amplified by PCR using the following primer pairs; Xlhbox-8: U: AAGGACAGTGGACAGATG D: GGA TGAAGTTGGCAGAGG, IFABP: U: GGAAGGTT GACAGAAGTG D: CCAAGAAGTTGGTTGTCC, Xsox17α: U: GGACGAGTGCCAGATGATG D: CT GGCAAGTACATCTGTCC, Xsox17β: U: GTCATG GTAGGAGAGAAC D: TCTGTTTAGCCATCA CTGG, HNF1β: U: GCAGCAGGAACTCCTCAA D: TGGTGGCCATTGGTGAGA, Endodermin: U: TATTCTGACTCCTGAAGGTG D: GAGAACTGC CCATGTGCCTC, Goosecoid: U: CACACAAAGT CGCAGAGTCTC D: GGAGAGCAGAAGTTGG GGCCA, brachury (Xbra): U: GCTGGAAGTATG TGAATGGAG D: TTAAGTGCTGTAATCTCTT CA, Xshh: U: ACTGCATAGCAGGAGGCCAA D: AAAGACCTGCGACCAGGGGA, Xchh:U: GAT GGACACCACGCTCACGAC D: TTGCACCTGAG TGCCATTCAC, ODC: U: GGAGCTGCAAGTTG-GAGA D: CTCAGTTGCCAGTGTGGTC. Samples were denatured for 3 min before cycling through 1 min at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. Standard PCR cycles (25) were used for primer sets except for ODC (20). Five micro liters of each PCR product was resolved by 2% agarose gel electrophoresis and observed with ethidium bromide.

Whole-mount in situ hybridization

Xchh in pBluescript II SK(−) were linearlized at either XhoI site (for the antisense probe) or XbaI site (for the sense probe) and were transcribed in vitro with either T3 or T7 RNA polymerase in the presence of digoxigenin-UTP. Albino *Xenopus laevis* embryos obtained as described above were processed for whole-mount in situ hybridization essentially following the method described by Harland (1991) with the modifications specified by Sasai et al. (1996). For clearing, the processed embryos were treated with clearing solution (2:1; benzyl benzoate: benzyl alcohol).

Results and discussions

Expression of Xchh in *Xenopus* embryo

We examined the localization of Xchh expression by whole mount *in situ* hybridization (Fig. 1). At early gastrula, Xchh RNA was initially detected in blastopore groove. At mid- and late-gastrula, its expression was observed in dorsal lip and pan-endoderm region and continued in pan-endoderm region throughout the neurula stage. From early tailbud, the expression in pan-endoderm region tended to be abundant at the anterior edge and then, in late tailbud, it localized strongly at foregut region. These results suggest that Xchh has important roles for the development of endoderm region, i.e. digestive organs. Interestingly, Xchh expression was identified at chordneural hinge which is posterior edge of mesodermal tissue, indicating that Xchh may also function in mesoderm tissue formation.

Existence of Xchh regulation factor in endodermal region

During animal development, differentiation of various tissues and cells are induced by signaling molecules provided from the neighboring cells and tissues. Typically, amphibian fertilized egg contains only two kinds of cells, animal and vegetal cells, which differentiate into ectoderm and endoderm tissues, respectively, and after fertilization, a part of animal cells are induced to differentiate into mesoderm tissues by factors secreted from vegetal cells. It is important to clarify whether Xchh expression would be activated endogenously or not. To analyze the existence of signals originated from the other tissues, it is useful that the tissues interested are excised from embryo and cultured separately.

The vegetal cells of mid-blastula stage embryo were excised and cultured solely in medium and the expression of Xchh was monitored by RT-PCR (Fig. 2). At the same time, the endoderm-specific marker genes, IFABP (Shi et al. 1994), Endodermin (Sasai et al. 1996), HNF1β (Bartkowski et al. 1993; Demartis et al. 1994), and Xsox17 and the mesoderm marker gene, shh were also tested. Ornithine Decarboxylase (ODC) was used as ubiquitous control. All genes examined here began to express in the isolated vegetal cells at the same developmental stages as those in whole embryo. At first, Xsox17 expression started from late blastula (stage 8.5) and endodermin and HNF1β did from gastrula (stage 12). XlHbox-8 (Wright et al. 1989) and IFABP expression began at later stage, tailbud (stage 23 and 28) when characters

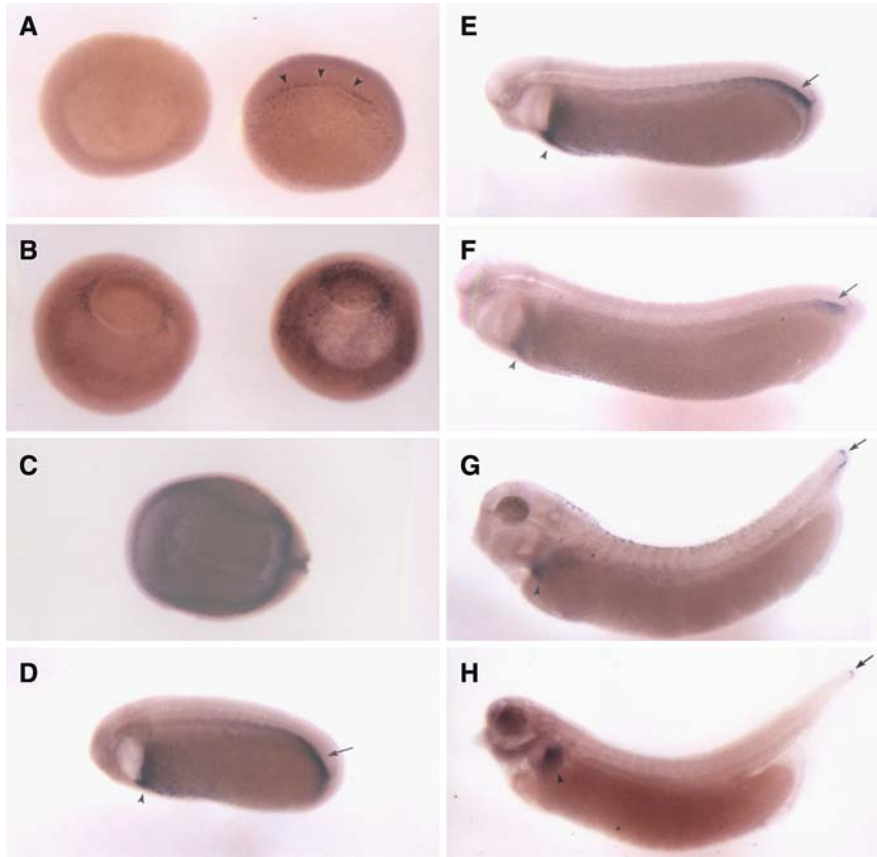

Fig. 1 Localization of Xchh mRNA during early development. (**a**) Vegetal views of gastrula embryos (left: stage9; right: stage10) with dorsal up. Xchh mRNA was localized at the blastopore groove (arrow head). (**b**) Vegetal views of gastrula embryos (left: stage11; right: stage12) with dorsal up. Xchh mRNA expressed endoderm cells migrating toward animal pole. (**c**) Top view of a neurula stage embryo (stage16) with anterior left. Xchh mRNA expression occurs throughout the endoderm region and archenteron roof. (**d**–**f**) Lateral view of a tailbud embryo (**d**: stage24; **e**: stage26; **f**: stage 28). Xchh mRNA expression was detected strongly anterior (arrowhead) and posterior (arrow) endoderm region. (**g**, **h**) Lateral view of a tailbud embryo (**g**: stage35; **h**: stage40). Xchh expression is detected in foregut (arrowhead) and neurochordal hinge (arrow)

of endoderm cells may be defined to specific digestive organs. These data indicated that the induction of these endoderm genes are not influenced with the other germ layer cells and regulated by the mechanism involved in its self. On the other hands, the maintenance of their expression seemed to need the signals from extra-endoderm tissues, because the expressions of Xsox17 and endodermin were different from those in whole embryo at later stages (stage 28 and 35). Transcription of Xchh mRNA in the isolated vegetal cells was started at early gastrula (stage 12), kept high level until early tailbud, and then decreased, as same as those in whole embryo did. From these results, it is suggested that Xchh expression is also regulated endogenously and the regulation factors for Xchh expression are involved in vegetal region cells of the fertilized egg.

Induction of Xchh mRNA by growth factors

Growth factors have important roles for the differentiation of embryonic cells in development. Activin/nodals are well known to direct vegetal cells to endoderm and mesoderm cells. BMP has ventralizing functions by competing with Activin/nodals. Activin and bFGF could differentiate animal cap cells to endoderm organs (Asashima et al. 1991, Jones et al. 1993, Sasai et al. 1996).

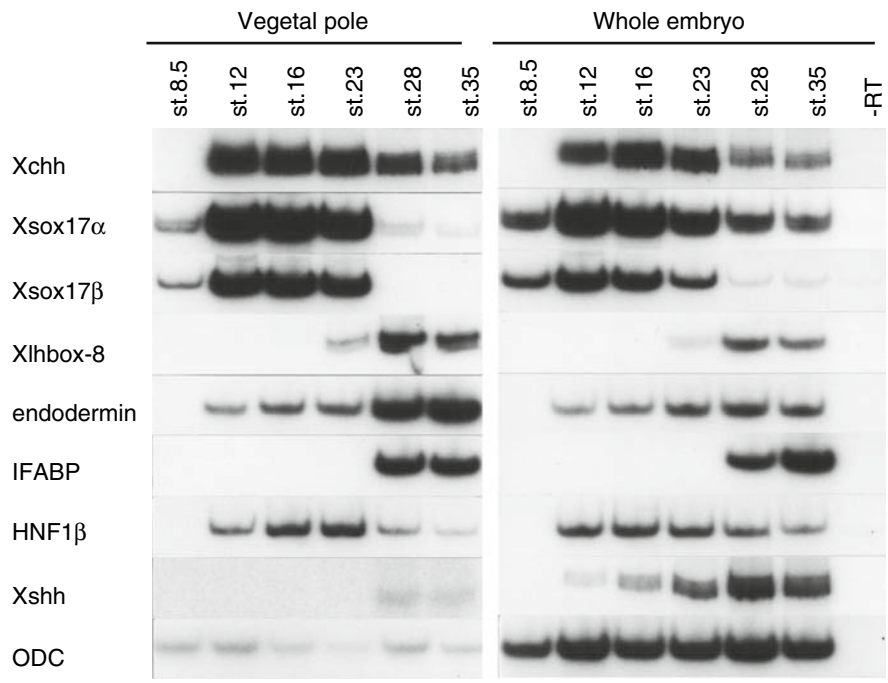

Fig. 2 Endodermal gene expressions in isolated vegetal cells. The vegetal cells dissected from mid-blastula embryo were cultured and used for RT-PCR. ODC is used as ubiquitous control

To examine whether the growth factor signals involved in vegetal cells could be related to the induction of Xchh expression or not, we analyzed the effect of dominant-negative receptors for various growth factors on Xchh expression in the isolated vegetal cells. Here, we used tAR, tBR and XFD, which are the truncated-form receptor of ALK7 (Hemmati-Brivanlou et al. 1992), BMPR2 (Suzuki et al. 1994), and FGFR (Amaya et al. 1991), for activn, BMP and bFGF signaling, respectively. Because they possess only their extra cellular ligand binding domains and membrane domains, they are able to inhibit their ligand signals dominantly. Translatable RNAs for tAR, tBR and XFD were synthesized in vitro and injected into fertilized egg. At blastula stage, vegetal cells were dissected from mid-blastula embryos (stage 8.5), cultured solely, and used for RT-PCR analysis. As shown in Fig. 3, Xchh expression was inhibited apparently by tAR RNA injection, while they were affected by neither tBR nor XFD. This suggests that the ligand of ALK7 is required for induction of Xchh in endoderm cells.

ALK7 receptor had been isolated and identified as activin receptor, but thereafter, various factors belonging to TGFβ superfamily are also reported to be capable to bind to ALK7 and they are called as activin/nodal factors. Among them, as the factors defined to exist in early *Xenopus* embryo, Vg1 (Yisraeli and Melton 1988), ActivinβB (Dohrmann et al. 1993), Xnr1/2/4/5/6 and Derrière (Sun et al. 1999) have been reported. Vg1 is maternally inherited substance localized at vegetal region and ActivinβB, Xnr1/2/4/5/6 and Derrière are expressed zygoticaly after fertilization. To confirm that activin/nodal factors can induce Xchh expression, we used the animal cap cells of embryo. The animal cap cells, which are destined to ectoderm cells, especially epidermis, in vivo, can differentiate to various types of cell in response to differentiation inducing factors. The animal cap cells injected with BVg1 mRNA, which is constitutive-active form of Vg1, were dissected from mid-blastula embryo and their RNAs were subjected to RT-PCR analysis. Vg1 induced Xchh expression extensively in animal cap cells (Fig. 4). Furthermore, the transcription of Xchh mRNA was activated by treatment animal cap cells with both activin and Xnr1 (data not shown). Combining with the results described above, it is concluded that activin/nodal factors are candidates to regulate the Xchh expression in embryonic endoderm region.

Xchh is induced by Xsox17

Because in endodermal differentiations, *Xenopus* Nodal-Related factor (Xnr) is induced by maternal

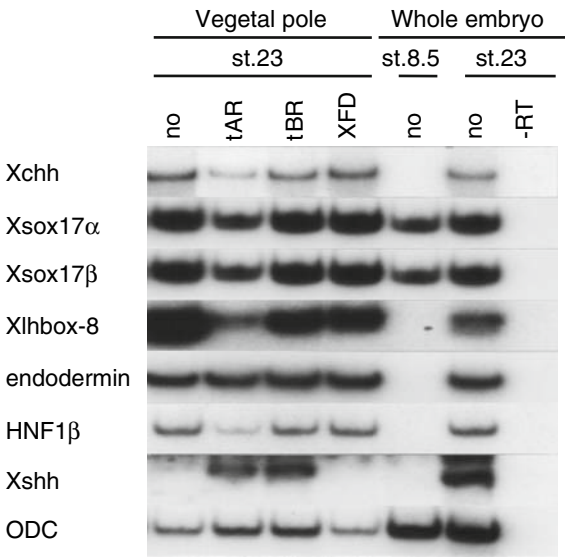

Fig. 3 Xchh expression depends on TGFβ signals. The vegetal cells were dissected from the embryos injected with dominant negative receptors for activin (tAR), BMP (tBR) and bFGF (XFD), cultured and used for Xchh expression analysis. In this experiment, to clarify the expression level of Xlhbox-8, 28 cycles was carried out for Xlhbox-8. no: not injected. –RT: RT reaction without reverse transcriptase

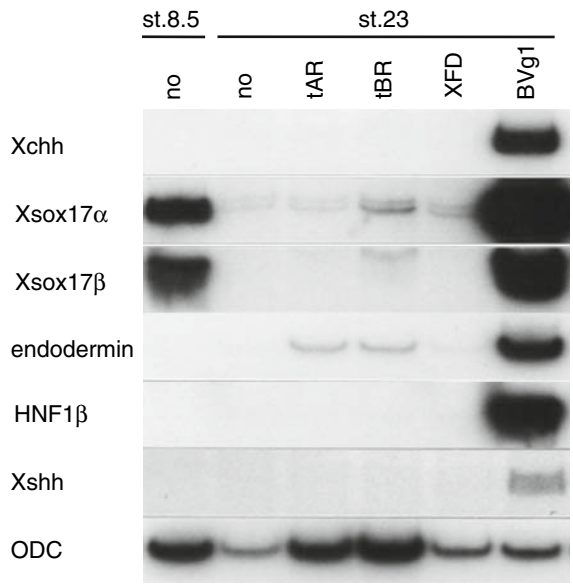

Fig. 4 Inductions of endoderm gene expressions in animal cap explants by activin/nodal factors. The animal cap cells were dissected from the embryos injected with the mature form of Vg1 (BVg1) mRNA, cultured and used for Xchh expression analysis. Dominant-negative receptors for activin (tAR), BMP (tBR) and bFGF (XFD), were also used here. no: not injected. –RT: RT reaction without reverse transcriptase

T-box transcription factor VegT, the transcriptional activation of Xchh is assumed to be under controls which started from VegT through Xnrs pathway. Some endoderm-specific transcription factors; homeobox genes, Mix/Mixer/Milk/Bix, HMG-domain factor, Xsox17α/β, and zinc-finger protein, GATA4/5/6, have been reported that they are also governed by regulators initiated from VegT through Xnrs. So, we examined which transcription factor can activate Xchh expression by using animal cap cells. The animal cap cells injected with *in vitro* synthesized mRNA for VegT, Mixer, Bix1-4, Mix1/2, and Xsox17 were dissected from mid-blastula embryo and their RNAs were subjected to RT-PCR analysis. As shown in Fig. 5a and b, VegT, Bix1, Mixer, and Xsox17 induced Xchh mRNA transcription in animal cap cells, while Bix2-3 and Mix1-2 did not. HNF1β, direct target of Xsox17 (Clements et al. 2003) and specific marker of foregut had no potential to activate Xchh expression (data not shown). Xsox17 has two homolog, α and β, and both of them were able to induce Xchh expression. Mixer is a transcription factor containing homeobox and Smad interacting motif and was reported that it has an inducing activity of Sox17 expression downstream of TGFβ signaling. Bix1 also activated Xchh expression (Fig. 5b). Bix1 is known as a direct target of Xbra which is the mesoderm inducing T-box transcription factor and moreover, BMP-responsive factor. Because Xchh in endoderm was not controlled by BMP signaling (Fig. 2) in endoderm cells, Xchh expression may not be under Bix1. From these results, it is suggested that Xchh expression in *Xenopus* endoderm cells is regulated by Sox17α/β downstream of maternal transcription factor VegT.

Xchh is one of direct targets of Xsox17

We examined the effect of the constitutive-negative form of Xsox17α (Xsox17α-en) which contained Engrailed-repression domain on the induction of Xchh by VegT and Xnr1. When mRNA for Xsox17 conjugated with Engrailed repression domain was co-injected with either VegT or Xnr1, Xchh expression induced by VegT and Xnr1 in the dissected animal cap cells were decreased (Fig. 6a). This indicated that the activation of Xchh expression through VegT

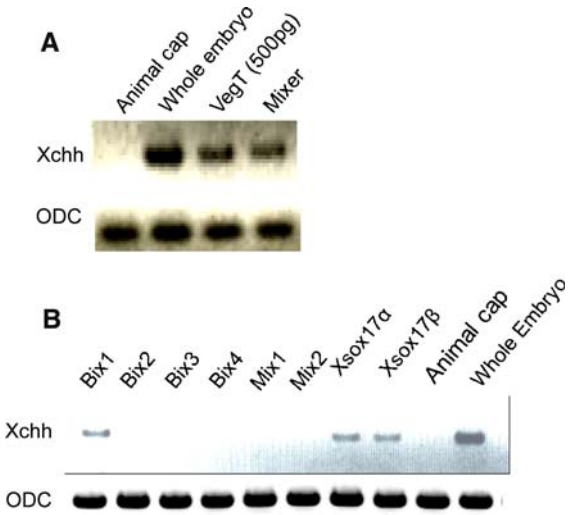

Fig. 5 Effects of endodermal transcription factors on Xchh expression. (**a**, **b**) The animal cap cells were dissected from the embryos injected with VegT, Mixer, Bix1-4, Milx1,2, and Sox17α/βmRNA (100 pg), cultured and used for Xchh expression analysis

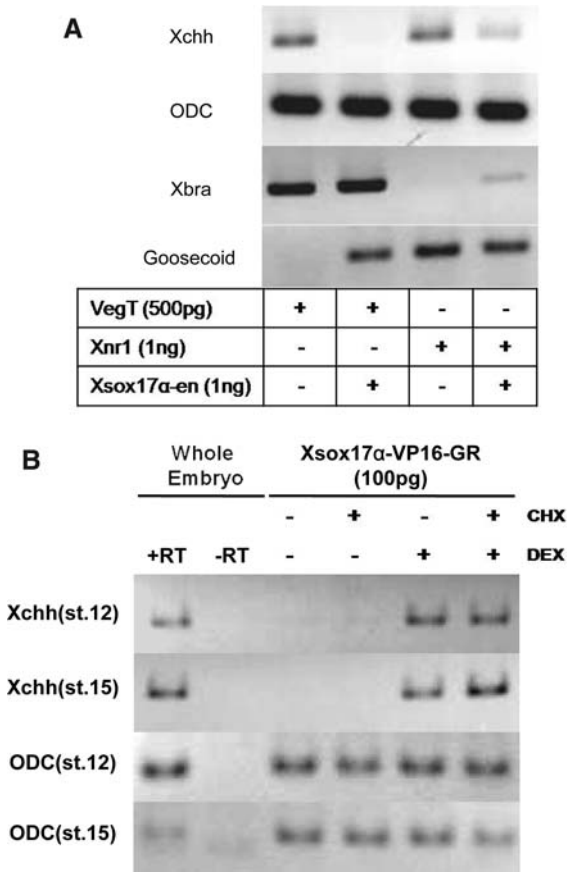

Fig. 6 Induction of Xshh expression by Sox17. (**a**) The animal cap cells were dissected from the embryos injected with VegT or Xnr1 with or without Xsox17α-en, cultured and used for Xchh expression analysis. (**b**) The animal cap cells were dissected from the embryos injected with Xsox17α-VP16-GR and cultured in medium only, medium with 10^{-6} M dexamethasone (DEX), medium with 10μg/ml cycloheximide (CHX), or medium with both 10^{-6} M dexamethasone and 10 μg/ml cycloheximide. After 5 h culture, the cells were used for Xchh expression analysis. −RT: RT reaction without reverse transcriptase

signaling cascade of RNA comprehend Sox17 activity. Interestingly, the co-injection of Xsox17α-en construct with VegT/Xnr1 caused to expression of mesodermal marker gene, brachury (Xbra) and goosecoid (Fig. 6a). This phenomenon coincides with the reports in which the repression of Xsox17 activity in endoderm cells caused to recovery of mesodermal factor (Engleka et al. 2001).

It was reported that HNF1β, endodermin, and HNF3α/βin endoderm cells are directly controlled by Xsox17 (Clements et al. 2003). As shown above, Xchh expression in *Xenopus* endoderm cells might be regulated by Xsox17α/β, but it is not proved whether its effect is direct or not. We examined the effect of Xsox17 on Xchh expression by using Xsox17α-VP16-GR construct. VP16 domain is a transcription activation domain of VP16 and GR domain is an active motif of glucocorticoid receptor. When cells introduced with transcription factor containing these two domains is exposed to dexamethasone (DEX), the transcription factor introduced acts as the dominant active transcription factor. Animal cap cells injected with Xsox17α-VP16-GR RNA were dissected and cultured with or without DEX. While no Xchh expression was observed without DEX, the induction of Xchh mRNA was occurred in animal cap cells cultured with DEX (Fig. 6b). Moreover, the induction by DEX was detected under existence of cycloheximide (CHX) which is a translation inhibitor, indicating that the induction of Xchh by Xsox17 does not need a new protein synthesis. From these results, it was concluded that Xchh regulation in *Xenopus* endoderm development is governed by the signaling cascades originated from the maternal inherited transcription factor VegT and its expression is initiated by Xsox17 direct action.

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